REPORT DOCUMENTATION PAGE

Form Approved
OMB NO. 0704-0188

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	21 November 2005	Final Report

4. TITLE AND SUBTITLE
Testing of WW-85

5. FUNDING NUMBERS
W911NF-05-1-0016

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Inotek

8. PERFORMING ORGANIZATION REPORT NUMBER
46981

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U. S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING / MONITORING AGENCY REPORT NUMBER
46981.1-LS-DRP

11. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

12 a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

12 b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)
See attached.

14. SUBJECT TERMS

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OR REPORT	18. SECURITY CLASSIFICATION ON THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	**UNCLASSIFIED**	**UNCLASSIFIED**	**UL**

NSN 7540-01-280-5500

Standard Form 298 (Rev.2-89)
Prescribed by ANSI Std. 239-18
298-102

A. PREFACE

The current document provides information on the development of WW-85, an ultrapotent catalytic anti-oxidant, as a radioprotectant countermeasure. Radiation injury induces the generation of multiple reactive oxygen and nitrogen radicals in living tissues, which then trigger oxidative, peroxidative, nitrosative and other forms of damage to proteins, lipids and nucleic acids in the cells. Data in the literature indicate that neutralization of these oxidant and free radical reactions and chain reactions is of protective value during radiation injury. However, the practical hurdles that hamper the introduction of a clinically useful anti-oxidant are that most available anti-oxidants (such as Vitamin C, Vitamin E, thiol-based compounds) are relatively slow reacting and do not neutralize fast-reacting species. In addition, effective concentrations of these compounds are difficult to be reached in living tissues. Preclinical data with radioprotectant anti-oxidants (amifostine, melatonin) demonstrate a relatively modest effect.

Our hypothesis is that an ideal anti-oxidant radioprotectant needs to be (1) fast-reacting; (2) catalytic, so that it does not get permanently consumed in the reaction; (3) non-selective, in the sense that it would react with both oxygen- and nitrogen-derived reactive species. In addition, a compound suitable for human use would need to be bioavailable, would need to have appropriate tissue distribution, and appropriately safe for human use. Currently, there are no such compounds available for clinical use.

The current document provides data showing that WW-85, a catalytic broad-spectrum anti-oxidant may fulfill the basic requirements for a development candidate as a broad-spectrum catalytic anti-oxidant for the purpose of radioprotection.

With respect to the general background of the application, we refer to the following review articles: Anderson, 1996; Liaudet et al., 2002; Cadet et al., 2003; Szabo, 2003; Harris et al., 2003; Slupphaug et al., 2003; Fortini et al., 2003; Dubrova et al., 2003; Kadhim et al., 2004; Mothesill et al., 2004. With respect to prior data with Inotek's earlier generation catalytic anti-oxidants, we refer to the following articles: Szabo et al., 2002; Mabley et al., 2002; Bianchi et al., 2002; Pacher et al., 2003; Naidu et al., 2004; Sugawara et al., 2004.

B. CONCEPTUAL BACKGROUND

B1.INTRODUCTION: SOURCES AND ACTIONS OF RADIATION-INDUCED RADICALS

It is well known that oxidants and free radicals, generated in response to ionizing radiation, play important roles as initiators and effectors of radiation injury. Fairly recent evidence suggests that cells are endowed with cytoplasmic amplification mechanisms involving reactive oxygen (ROS) and nitrogen (RNS) species and that these systems are responsible to damage and positive feedback cycles of radiation injury (via various signal transduction and amplification processes. The distinction between primary free radical-mediated injury processes, and the subsequent, later free radical mediated injury is important, both from a basic research (mechanistic) standpoint, and from a practical standpoint, because if free radicals are only important in the acute stage of radiation injury, then free radical and oxidant neutralizing strategies would only be expected to work in a pretreatment regiment. Alternatively, if irradiation induces a self-amplifying series of subsequent events, which are also governed by oxidants and free radicals, then one would expect a significant therapeutic window of opportunity for NOS/RNS neutralizing agents in subjects subjected to whole-body irradiation. The potential sources and sensors for these ROS/RNS and the evidence for amplification mechanism are summarized in the subsequent section.

B.1.1. Radiation-Induced Generation of ROS/RNS The primary radical generated as a consequence of initial ionization events is the hydroxyl radical (·OH), which is short-lived and only diffuses about 4 nm before reacting with one of its many biological targets. Of the secondary ROS, superoxide (O_2^-) and hydrogen peroxide (H_2O_2), the latter can react via Fenton chemistry with cellular metal ions to produce additional ·OH.

Less information is available about RNS that may also be produced after irradiation and may be a consequence of radiation-induced stimulation of nitric oxide synthase activity in cells that express this enzyme. Reaction of nitric oxide with O_2^- results in the formation of peroxynitrite, a membrane-permeant and relatively stable RNS. When protonated, peroxynitrite isomerizes to *trans*-peroxynitrous acid, which can cause protein and DNA damage similar to ·OH. Recent data indicate that peroxynitrite **generation plays an important pathogenetic role in mice subjected to whole-body irradiation**. The reactivity and toxicity of peroxynitrite, are, therefore, discussed, in detail, in a subsequent separate section.

B.1.2. Cellular Amplification of Radiation-Induced Generation of ROS/RNS Indirect evidence for an extranuclear radical amplification mechanism has come from studies with high-LET particles. For example, bone marrow progenitor cells exposed to neutrons show both an enhanced ability to oxidize a fluorescent probe for ROS/RNS and increased 8-hydroxy-2-deoxyguanosine levels indicative of oxidative DNA base damage. Direct evidence for cytoplasmic ionization events affecting nuclear processes has come from studies in which alpha-particle irradiated individual cells that permits selective irradiation of the cytoplasm or nucleus. Irradiation of the cytoplasm was shown to be mutagenic but not cytotoxic. The major class of mutations was similar to that generated spontaneously and thought to arise from DNA damage due to endogenous production of ROS/RNS and differed from that induced after irradiation of the nucleus.

Studies with fluorescent dyes demonstrated generation of ROS/RNS in cells within 15 min after irradiation with less than one alpha particle per cell. The intracellular production of ROS/RNS was 50-fold greater than the extracellular production. The intracellular source was inhibited by diphenyl-iodonium, an inhibitor of flavoproteins, suggesting the involvement of a plasma membrane NADPH oxidase. However, flavoproteins are also localized to the mitochondria and endoplasmic reticulum, possible alternative cellular organelles that generate ROS/RNS.

The radiation-induced stimulation of secondary production of ROS/RNS at the single-cell level and demonstrated in several human carcinoma cells that ROS/RNS were generated within seconds after radiation exposure. The use of several inhibitors pointed to the mitochondria as the source for ROS/RNS, a conclusion also supported by the observed simultaneous transient depolarization of the mitochondrial membrane potential.

B.1.3. Consequences of Radiation-Induced Generation of ROS/RNS Some ROS/RNS, e.g. H_2O_2, nitric oxide and peroxynitrite, are membrane-permeant and are sufficiently stable to diffuse significant distances within cells, facilitating their interaction with biological molecules, including proteins, lipids and DNA. These are capable of inducing various pathways of direct injury. In addition, they can be involved in the regulation of cytoplasmic signal transduction pathways are protein SH residues. The interconversion of oxidized and reduced Cys represents a reversible controlling element in the regulation of protein functions. For example, H_2O_2 or more reactive ROS have been shown to reversibly oxidize a Cys to a cysteine sulfenic acid in the catalytic site of Tyr phosphatase 1B, resulting in transient inhibition of phosphatase activity. A possible consequence of this is the enhanced phosphorylation and activation of target proteins, such as EGFR (also known as ErbB1). Similarly, nitric oxide stimulates guanine nucleotide exchange on RAS by *S*-nitrosylation of a critical Cys. Irradiation of cells also induces lipid peroxidation and changes in membrane structure in intact cells. How these structural changes modulate membrane function, e.g. activation of EGFR, and the cellular response to radiation are not yet known in sufficient molecular detail. However, they provide potential mechanisms, through free radical propagation or changes in membrane fluidity, for the amplification of localized perturbations of the membrane structure to signals throughout the cell. In the next section, we will examine some of the cell injury and defense mechanisms involved in the **acute cellular effects** of ROS/RNS after irradiation. In the subsequent question, we will examine some of the **cellular amplification pathways** involved in the action of ROS and RNS after irradiation.

B.2. ACUTE CELLULAR EFFECTS OF FREE RADICALS AND OXIDANTS IN RESPONSE TO IONIZING RADIATION

As mentioned above, radiation injury to living cells is, to large extent, due to oxidative stress. Reactive oxygen species (ROS) and free radicals induced by partial reduction of oxygen (O_2) react with cellular macromolecules (i.e., nucleic acids, lipids, proteins, and carbohydrates) and damage them. The interaction of ionizing radiation with living cells induces a variety of reaction products and a complex chain reaction in which many macromolecules and their degradation products participate. Major biomarkers of oxidative damage to living cells are (i) lipid peroxidation (LPO) products, comprising volatile hydrocarbons measurable in exhaled air, such as ethane and penthane, and isoprostanes and aldehydic products measurable in tissues and body fluids; (ii) DNA-hydroxylation products (e.g., 8-hydroxy-2'-deoxyguanosine (8-OHdG)) and microscopic indices of damage such as chromosomal aberrations and micronuclei; and (iii) protein hydroxylation products such as oxidized amino acids.

B.2.1. Oxidative Damage to DNA Ionizing radiation is a well-established carcinogen due to the resulting oxidative damage, and the molecule most often reported to be damaged by this physical agent is DNA. Interactions of ionizing radiation with DNA consist of the direct ionization of DNA (direct effect) and its reaction with surrounding water molecules (the indirect effect), followed by DNA destruction by the induced radicals (•OH, e–• and, to much lesser extent,

H•). A wide variety of biochemical consequences of radiation-induced damage to DNA occurs because of free-radical attack.

About 60%–70% of the acute cellular DNA damage produced by ionizing radiation is estimated to be caused by •OH, formed from the radiolysis of water. •OH are considered the most damaging of all free radicals generated in organisms. All biological molecules are targets for •OH, and this radical reacts with them at a very high rate, exceeding $10^9\ M^{-1}\ s^{-1}$. Thus, the intracellular diffusion distance of •OH is on the order of Ångstroms, and the damage it produces is essentially at the site where it was generated. Of the DNA damage produced by •OH, oxidized bases, abasic sites, DNA-DNA intrastrand adducts, DNA single and double strand breaks, and DNA-protein cross-links are the most significant. These occur primarily by interaction of free radicals with DNA bases and, to a lesser extent, with DNA sugars.

Among bases and, generally among nucleic acid components, guanine is the most susceptible DNA target for oxidative reactions mediated by •OH and other free radicals, and it exhibits the lowest ionization potential. Thus, one of the most mutagenic lesions, and the most abundant lesion formed in irradiated chromatin is 8-hydroxyguanine. Once formed, this product can be repaired by several mechanisms. Under laboratory conditions, one of the most common and abundant measurable oxidative DNA base adducts is 8-OHdG. This is reported to be a key biomarker related to carcinogenesis.

The interaction of free radicals with sugar moieties leads to the cleavage of the sugar-phosphate backbone of DNA followed by single-strand breaks that undergo repair processes relatively easily. On the other hand, double-strand breaks have more serious consequences. Double-strand breaks are well correlated with the cytotoxic effects of ionizing radiation and are considered the primary lesion involved in cellular death.

If DNA repair mechanisms, which are induced after exposure to ionizing radiation, are inefficient, the damaged DNA strands that are copied during replication lead to mutagenesis and carcinogenesis. The damaging effects of ionizing radiation lead to cell death and are associated with an increased risk for numerous genetically determined diseases.

Among other indices of DNA damage caused by ionizing radiation are chromosome aberrations and micronuclei formation; these are apparent when irradiated cells are observed microscopically.

When overviewing the experience with various radiation protectors, one can conclude that even a partial success in neutralizing hydroxyl radicals may result in significant therapeutic effect, and pretreatment approaches can be effective, at least as acute cytoprotectants. This may be either related to the fact that (a) acute radiation-induced effects are not primarily due to DNA damage, and/or due to the fact that (b) free radicals and oxidants induce secondary signal transduction processes, which lead to amplification and exacerbation of the injury.

When overviewing published data with radioprotectants, one can conclude that there is a window of opportunity even in the post-treatment regimen, at least for prolongation of the acute survival. Indeed, data with mice subjected to lethal whole-body irradiation indicate, that post-treatment anti-oxidant strategies can be effective (at least in delaying acute mortality).

B.2.2. Oxidative Damage to Biological Membranes Besides DNA, lipids and proteins are also attacked by free radicals induced by ionizing radiation. The initiated chain reaction caused by ionizing radiation leads to the formation of a variety of degradation products in biological membranes including products of lipid breakdown. These degradation products induce changes in membrane structure and function that are exaggerated by the accumulation of free radical–mutilated proteins. These degradation products make membranes more rigid (less fluid) and furthermore, after they migrate out of the membrane they react with proteins and nucleic acids, thereby contributing to DNA damage and mutagenesis. Significant changes in structure and function of membranes result in cell death *via* necrosis and/or apoptosis.

In a lipid-rich environment, as in membranes, both the phospholipid acyl chain and phospholipid backbone as well as cholesterol are subject to radiation damage. However, polyunsaturated hydrocarbon moieties of the phospholipids are particularly sensitive to attack by reactive oxygen species. Exposure of liposomes to gamma-radiation confirms the particular susceptibility of polyunsaturated acyl chains to oxidative damage. Thus, the radical chain reactions induced by ionizing radiation result in the production of hydroperoxides at the unsaturated sites of lipid acyl chains; hydroperoxide residues change the hydrophobic interactions between adjacent chains of phospholipids allowing easier penetration of water molecules to the most external portion of the bilayer, thereby altering the electric constant across the bilayer. The altered gradient of water concentrations throughout the membrane has relevant biological consequences since it changes membrane substructure directly and indirectly leads to the degradation of lipids and proteins. Membrane damage caused by ionizing radiation is of particular importance because these altered molecules are not repaired after exposure either to low fractionated doses or to a single acute dose of ionizing radiation.

A commonly measured parameter of lipid damage after ionizing radiation exposure is thiobarbituric acid reactive substances (TBARS). These products, which include malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA), result from the interaction of free radicals with polyunsaturated fatty acids (PUFA). The formation of TBARS as an index of lipid breakdown is a complex process which is due to the high susceptibility of PUFA to free radicals, the production of lipid peroxyl radicals (LOO•), endoperoxides and hydroperoxides, and, thereafter, the propagation of a chain reaction leading to the extensive lipid damage. Another biochemical parameter used as an index of radiation-induced damage to membranes is the level of isoprostanes. The evaluation of oxidative damage to membranes by fluorescence spectroscopic methods is often used also.

Alterations in the properties of biological membranes caused by ionizing radiation are due to complex processes involving both lipids and proteins. These changes are likely to contribute both to the acute effects of radiation, and to the delayed amplification effects. There is no consensus as to whether damage to proteins vs. lipids is of primary importance in the acute effects of radiation toxicity.

B.2.3. Radiation Damage to Mitochondria: Irradiation-Induced Release of Mitochondrial ROS Recent data indicate, that, contrary to previous thinking, a significant portion of the free radicals and oxidants are produced in the mitochondria and lysosomes (as opposed to the nucleus). This ROS generation was apparent several hours after irradiation in lymphocytes *in vitro*. These data are important because they indicate the existence of free radical-mediated amplification processes. If mitochondrial ROS production plays a role in the death or irradiated cells, one would expect that these processes represent on-going processes, and would offer the opportunity for post-treatment interventions (see also below). Part of the mechanism relevant to these processes may be related to caspases. Although caspases, historically, are viewed as effectors and executioners of apoptotic nuclear cleavage processes, recent work demonstrates that caspases also exert deleterious effects on mitochondrial structure and function and induce ROS generation from mitochondria. One should note here, that irradiation induces DNA damage not only in the nucleus, but also in the mitochondria, but the potential relationship between irradiation-induced mitochondrial DNA damage and irradiation-induced mitochondrial ROS production has not yet been elucidated in detail.

B.2.4. Physiological and Pharmacological Defense Against Oxidative Stress A variety of defense and repair mechanisms exist in living cells to protect against oxidant species. The antioxidative defense system is composed of methods to (i) transfer sensitive material to compartments better protected from the action of reactive species, (ii) complex transition metals, a potential source of electrons, thereby rendering them unreactive, (iii) inhibit vulnerable processes such as DNA replication, (iv) repair damaged molecules, (v) initiate apoptosis, and, possibly the most important considering the lability to internal and external modifying factors, (vi) activate anti-oxidant enzymes and finally (vii) use a variety of direct free radical scavengers. Of the enzymes involved in anti-oxidative defense, particularly well documented are the antioxidative properties of the superoxide dismutases (SOD), glutathione peroxidases (GSH-Px), and catalase (CAT).

Since the amount of DNA damage caused by ionizing radiation is correlated with the intensity of oxidative stress, with the efficacy of defense mechanisms that metabolize toxic intermediates and with intrinsic repair mechanisms, there are several potential means to reduce macromolecular damage due to ionizing radiation. Since radiation-induced cellular DNA damage is primarily attributed to the damaging effects of free radicals, molecules with direct free radical scavenging properties can serve as effective radioprotectors. The involvement of free radical scavengers in protecting against radiation damage is emphasized by the observation that whole body irradiation decreases the total anti-oxidant capacity of the organism and depletes the levels of known anti-oxidants such as ascorbic acid and uric acid as they are used in the scavenging of free radicals and oxidants.

A number of different substances have been examined as to their radioprotective effects against cellular damage caused by ionizing radiation. Several anti-oxidants have been proven to be efficient protectors against ionizing radiation (e.g., 5-aminosalicylic acid and ascorbate against 8-OHdG formation in calf thymus DNA, flavonoids, especially luteolin, against lipid peroxidation, and micronuclei formation in mouse peripheral blood erythrocytes and panthotenol against lipid peroxidation in rat liver. Amifostine and melatonin have also been demonstrated to act as radioprotecting agents both *in vitro*, in cells subjected to various types of irradiation, and in murine studies *in vivo*, in mice subjected to lethal doses of gamma-irradiation.

Melatonin. Melatonin is a fairly effective natural anti-oxidant, with effects on hydroxyl radical, peroxynitrite and other reactive oxygen and nitrogen species. The radioprotective actions of melatonin were shown first in Swiss ND4 mice: the animals exposed to 950 cGy of whole body radiation died 12 days later, whereas when mice were pretreated with

melatonin (1.076 mmol/kg body wt), 43% of the irradiated mice survived at least 30 days after treatment. A similar survival study observed that the exposure of mice to 815 cGy of ionizing radiation resulted in only a 45%–50% survival rate after 30 days; pretreatment with melatonin at a dose of 125 mg/kg body wt slightly increased the survival rate to 60%, whereas melatonin at a dose of 250 mg/kg body wt significantly increased survival to 85%. The effects of melatonin were associated with protection against the increases in the levels of MDA in ovaries and plasma after radiation exposure and protective effects were also observed against changes in guanine bases. Melatonin provides proof of principle that anti-oxidant compounds may be effective in protecting against lethal radiation injury. Much less clear is whether melatonin and its analogs, in the doses used in the animal studies, would be ever applicable to the human situation. In addition, one must note that melatonin loses its effectiveness when given in post-treatment regimen, and when melatonin, in very high doses, is used in hyperagressive irradiation regimens (such as the one we use in our preliminary studies, where the rate of radiation is as high as 5 Gy/min), the protective effect of melatonin, even when given at high doses and as a pretreatment, becomes minor.

Amifostine. Amifostine (Ethyol), an inorganic thiophosphate, is a selective broad-spectrum cytoprotector of normal tissues that provides cytoprotection against ionizing radiation and chemotherapeutic agents, thus preserving the efficacy of radiotherapy and chemotherapy. Amifostine, an inactive pro-drug, is transformed to an active thiol after dephosphorylation by alkaline phosphatase found in the normal endothelium. The cytoprotective mechanism of amifostine is complicated, involving free radical scavenging, DNA protection and repair acceleration, and induction of cellular hypoxia. Intravenous administration of amifostine 740-900 mg/m2 before chemotherapy and 250-350 mg/m2 before each radiotherapy fraction are widely used regimens. The US FDA has approved the use of amifostine as a cytoprotector for cisplatin chemotherapy and for radiation-induced xerostomia and as such, it represents one of the few clinically approved radioprotecting agents. Amifostine in reducing radiation-induced mucositis and other toxicities, and it appears that it does not interfere with the primary anti-tumor effect of radiation. Amifostine has also been tested in various animal models of whole-body irradiation, and at high doses (e.g. 300 mg/kg it appears to be effective, in pretreatment regimens, to improve survival of the animals and to reduce acute radiation-induced mortality. However, the general consensus in the field is that the efficacy of amifostine is not robust enough, and the dose required in human beings is not achievable enough to be seriously considered for the purpose of the current RFA.

B.3. FREE RADICALS, OXIDANTS AND SIGNAL TRANSDUCTION PATHWAYS IN THE CONTEXT OF AMPLIFICATION OF THE RADIATION-INDUCED CELLULAR RESPONSES

B.3.1. Cytokines and membrane receptors Ionizing radiation activates cytokine receptors, such as EGFR and tumor necrosis factor receptor (TNFRSF1A, formerly known as TNFR) with consequences currently indistinguishable from the effects of the physiological cytokine/growth factor ligands. The downstream effects of receptor activation are the initiation and amplification of signals along growth and stress pathways, involving MAPK, PI3 kinase and MAPK8, resulting in modulation of the proliferation and survival states of cells.

B.3.2. Delayed Activation of Cytoprotective Response Cascades The term cytoprotective describes the summation of responses, which favor cell survival and may span from enhanced repair functions to stimulation of proliferation. These responses may be initiated at the level of the plasma membrane through activation of ERBB receptor tyrosine kinases and other related molecules. The radiation-induced activation of EGFR has been linked mechanistically to enhanced signaling through critical components of the MAPK and MAPK8 pathways. The radiation-induced activation of EGFR at doses between 1 and 5 Gy results in the activation of several immediate downstream effectors including RAS, PLC with production of diacylglyceride and inositol trisphosphate (IP3) and diacylglyceride-mediated activation of protein kinase C (PKC) and IP3-induced release of Ca2+ from intracellular stores. RAS-GTP recruits RAF1 to the plasma membrane and RAF1 primarily signals along the MAPK cascade involving MAP2K1/2 and p90S6 kinase. MAPK and p90S6 kinase regulate diverse targets in cells including transcription factors. RAF1 has also been shown to phosphorylate the anti-apoptosis protein BCL2, a modification that may counteract the anti-apoptosis function of BCL2. The importance of RAS lies in its potential cross-communication into the MAPK8 pathway. The critical role of MAPK as downstream effector of EGFR is demonstrated by the finding that inhibition of MAPK by PD98059 disrupts the cytoprotective response against radiation.

B.3.3. Delayed Activation of Cytotoxic Response Pathways Two forms of the tumor necrosis factor-alpha receptor have been characterized as CD4 (formerly known as p55) and TNFRSF1B (formerly known as p75). Both receptors can activate the lipid-cleaving enzyme acid sphingomyelinase (ASMase) with the production of ceramide. Ceramide, through the family of GTP-binding molecules RHO/RAC and RAS, leads to the activation of downstream signaling molecules MEKK1/2, which are analogous to RAF1 in the MAPK pathway. Active MEKK1 in turn can phosphorylate and activate MAP2K4/7 and MAP2K3/6. These molecules share considerable sequence similarity to MAP2K in the MAPK pathway. MAP2K4/7 signals to MAPK8, which phosphorylates the transcription factor JUN, and has been shown to facilitate apoptosis in certain cell types. For example, in several studies using cells of hematopoietic lineage, enhanced MAPK8 signaling has been closely associated with apoptosis in response to cytotoxic stimuli. In other nonhematopoietic cells, such as carcinoma cells, increased MAPK8 signaling can result in increased proliferation and protection from radiation and other cytotoxic stresses. Thus the precise role of MAPK8 activation in response to exposure of cells to radiation may vary substantially with the cell type analyzed. Alternatively, MAP2K3/6 may signal along a rescue pathway involving MAPK1 (formerly known as p38) reactivating kinase, which facilitates phosphorylation of heat-shock proteins and cell survival.

Ceramide and death receptor systems play important roles in modulating the effects of cellular stress responses on MAPK8 and MAPK signal transduction, as well as the impact of the former on apoptosis after radiation exposure. One source of ceramide results from cleavage of sphingomyelin by ASMase in cells exposed to IL1B, and the binding of death receptor ligands such as TNFRSF6 and TNF, to specific membrane receptors. A similar effect is mediated by radiation. Ceramide specifically targets ceramide-activated protein kinase (CAPK) and phosphatase (CAPP). CAPK can directly activate RAC1, a component of the stress pathway, and can also, through a process yet to be fully elucidated, activate RAF1, a component of the PRKC/RAF1/MAPK cytoprotective pathway. Thus ceramide may function as an upstream modulator of cytoplasmic kinase cascades. Immediate downstream targets of CAPP include novel isoforms of protein kinase C, e.g. PRKCZ, which are sensitive to ceramide but insensitive to diacylglyceride and phorbol ester. Downstream of RAC1/MEKK1 lies MAP2K4/7 and its target, the stress-activated protein kinase MAPK8, which has been implicated in apoptosis induced by TNF and other environmental stresses. Downstream targets of PRKC and RAF1 include MAP2K1/2 and MAPK, which activate anti-apoptosis signaling elements, including the CDK inhibitor CDKN1A and possibly through modulation of IKB and NFKB. As in the case of radiation-induced activation of EGFR receptor tyrosine kinase, the impact on cell survival of the balance between the relative signal intensities along the MAPK and MAPK8 pathways is not fully understood. The components of ceramide-mediated signaling underlie the cellular responses linked to radiation-induced apoptosis. One consequence of MAPK8 activation includes induction of cytokines, such as TNFSF6 and TNF, which bind to and activate a series of death receptors, including TNFRSF6, TNFRSF1A and TNFSF10. Activation of these death receptors ultimately culminates in cleavage/activation of effector caspases, which represent key components of the protease cascade leading to apoptosis. It is also possible that MAPK8 activation may initiate caspase activation through an alternative, death receptor-independent pathway.

The extent of apoptosis is also determined by the expression of pro- and anti-apoptosis genes of the *BCL2*/*BAX* family. Through a number of unidentified steps, CAPK, secondary to activation by ceramide, promotes apoptosis through a process that involves the pro-apoptosis proteins BAX and BAD. BAD exerts its function by binding to anti-apoptosis proteins such as BCL2 and BCL2L1, and preventing them from antagonizing cell death mediated by BAX. The mechanism by which pro-apoptosis proteins such as BAX and BAD promote and anti-apoptosis proteins such as BCL2 and BCL2L1 prevent cell death is not known with certainty, but that these events are linked to perturbations in mitochondrial function. For example, pro-apoptosis proteins induce loss of the mitochondrial membrane potential and release of the apoptosis-promoting proteins, cytochrome c and PDCD8, into the cytoplasm. Conversely, anti-apoptosis proteins such as BCL2 appear to act by preserving mitochondrial integrity. Release of cytochrome c into the cytoplasm leads to activation of a complex consisting of APAF1, cytochrome c, dATP and CASP9, which then cleaves and activates caspase 3. It remains to be determined whether components of the stress pathway such as MAPK8 can lead directly to mitochondrial damage/apoptosis caspase activation, or whether they act indirectly through the modulation of pro- and anti-apoptosis proteins.

The interest in mechanisms regulating apoptosis have stimulated investigation of the relationship between the activity of death pathways to the activation of the caspase cascade. The death receptors have been identified as plasma membrane-associated receptors. The signals are transmitted by the respective ligands, APOL, TNFSF6 and TNF, suggesting the involvement of signal transduction components from other response systems described above. The immediate downstream effectors of these death receptor pathways include various adapter protein complexes, including

Fas-associated death domain (FADD) and TNFRSF1A-associated death domain (TRADD) proteins. These complexes initiate responses of cytoplasmic promoters of apoptosis, notably activation of pro-caspase 8. Caspase 8 in turn activates caspases 1, 3 and 6. Caspase 8 may also act by inducing cleavage of the BCL2 family member BID, thereby exposing its pro-apoptosis BH3 domain. Thus activation of effector caspases through the TNFSF6/TNFRSF6/FADD/caspase 8 or the TNF/TNFRSF1A/TRADD/FADD/caspase 8 pathways can proceed independently of the cascade to apoptosis linked to mitochondrial dysfunction described above and activation of pro-caspase 9. The activation of caspase 1, 3 and 6 leads to degradation of a wide variety of cell death substrates, such as PARP and RB protein among many others, as well as activation of the DNA fragmentation factor (DFFA/DFFB), events that are essential for cellular disassembly and progression to apoptosis.

Many details regarding the relative role of the death receptor- and mitochondria-related pathways in radiation-mediated cell death remain to be elucidated. An important question to be resolved is whether radiation initiates cell death through activation of specific signaling/death receptor pathways, or possibly through generation of ROS, directly inducing mitochondrial damage with caspase activation. As already mentioned above, recent data indicate that caspases can feed back to the mitochondria and can be involved in the generation of delayed oxidant formation from a dysfunctional mitochondrial respiratory chain, thereby possibly participating in positive feedback cycles involving ROS/RNS mechanism in irradiated cells.

B.4. SPECIFIC ORGANS INVOLVED IN RADIATION INJURY: THE QUESTION OF PREVENTION OF DAMAGE VERSUS PROMOTION OF REGENERATION

One crucial issue that has not been discussed in the above sections is the specific organs involved in radiation-induced damage (radiation sickness, radiation-induced acute mortality). While whole-body irradiation, by definition, affects all cells of the body, certain cell types and certain organs are more sensitive than others, and are primarily contributing to the acute radiation induced damage and mortality. These organs are (1) the intestinal epithelium and (2) the bone marrow. The relative contribution of the two organs depends, among others, on the experimental model in question, the dose and the rate of the radiation used, the severity of the injury, and other modifying factors, as well as the presence or absence of various supporting therapies in the irradiated animals.

Bone marrow One must keep in mind that the immune system is composed of multiple cellular elements (not only the marrow), so it is not surprising that the effects of radiation on it are multiple and diverse. Profound immunosuppression is a normal consequence of whole-body radiation exposure, but regional radiation therapy can also result in systemic suppression. Immunosuppression is most often ascribed to lymphocytes being highly radiosensitive due to their proclivity to undergo radiation-induced apoptosis. Although lymphocyte killing is obviously one mechanism by which radiation affects the development of immunity, other mechanisms of immunomodulation are possible. As far as the extent of bone marrow toxicity goes, it is crucial to distinguish between eradication of bone marrow cells versus subsequent repopulation processes. In preclinical studies, exposure of mice to 7–15 Gy total body irradiation results in the death of most proliferating cells in the bone marrow, but some stem cells survive and repopulate the marrow. With higher doses of radiation, the number of surviving bone marrow stem cells is insufficient to repopulate the marrow, and the animal dies. Development of therapeutic approaches that would accelerate the repopulation process would be certainly of great importance.

Intestine Acute radiation induces bacterial translocation from the gut, followed by systemic infection and sepsis. In order to reduce the mortality after acute whole body irradiation, it is essential to control bacterial translocation. In many models of whole-body irradiation, bacterial translocation assays are used predictors of the overall survival outcome. One can question the relative importance of gut toxicity (as opposed to marrow toxicity) in the context of radioprotection in humans, inasmuch as in preclinical experiments, normally, antibiotics and other means of therapeutic management of the septic condition are not administered (as opposed to humans, where these supporting therapeutic options are available in the hospital setting. From a mechanistic standpoint, the issue of cytotoxicity vs. regeneration is crucial in the context of the gut (just as much as it is crucial in the context of marrow). After 8–14 Gy of total body irradiation, proliferating transit cells in the intestinal crypt are killed; but some stem cells survive. Surviving stem cells proliferate and give rise to transit cells that form regenerative crypts and eventually repopulate the mucosa. Higher doses of radiation kill more of the stem cells and, consequently, reduce the number of regenerative crypts.

B.5. PEROXYNITRITE: FORMATION, CYTOTOXIC ACTIONS AND POTENTIAL ROLE IN RADIATION INJURY

B.5.1. Peroxynitrite: formation and reactivity Simultaneous generation of nitric oxide and superoxide favors the production of a toxic reaction product, peroxynitrite anion (ONOO). In *in vitro* systems, the ratio of superoxide and NO determines the reactivity of peroxynitrite: excess NO reduces the oxidation elicited by peroxynitrite. The end-products of specific oxidative processes triggered by peroxynitrite can be detected *in vivo*, suggesting *in vivo* formation of peroxynitrite (see below). The oxidant reactivity of peroxynitrite is mediated by an intermediate with the biological activity of hydroxyl radical. This species is not hydroxyl radical per se, but rather, peroxynitrous acid or its activated isomer.

The decomposition of peroxynitrite to nitrate is intimately coupled with the oxidation chemistry of this species, and both reactions have been the subject of recent investigations and intense debate. Peroxynitrite and its conjugate acid are strong oxidants, capable of effecting one- and two-electron reactions akin to those of $HO\cdot$, nitrogen dioxide (NO_2), and nitrosonium cation. Oxidations of thiols, sulfides, transition metal complexes, deoxyribose, phenols and other aromatics by peroxynitrite have been described.

Peroxynitrite is a particularly effective oxidant of aromatic molecules and organosulfur compounds that include free amino acids and peptide residues. Cysteine and glutathione, which are significant components of anti-oxidant reservoirs, are converted to disulfides. Tyrosine and tryptophan undergo one-electron oxidations to radical cations, which are competitively hydroxylated, nitrated, and dimerized.

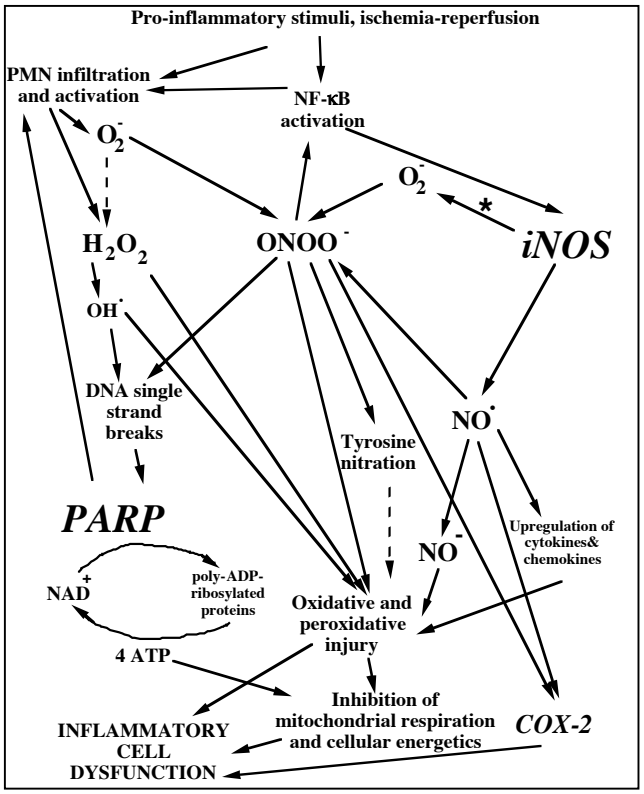
In *in vitro* systems, peroxynitrite is highly reactive. Its reported activities include a rapid oxidation of sulfhydryl groups and thioethers, as well as nitration and hydroxylation of aromatic compounds, including tyrosine, tryptophan and guanine. While the reaction with the sulfhydryl groups is likely to represent a direct reaction of peroxynitrite, the tyrosine nitration probably occurs through a $NO2^{+}$-like intermediate. The detection of 3-nitrotyrosine by analytical and immunological techniques has established that a marked increase in tyrosine nitration occurs in a wide variety of disease states.

The various reactions of peroxynitrite when occurring during the reaction of peroxynitrite with enzymes, macromolecules and lipids, have been shown to influence cellular functions. For instance, tyrosine nitration may lead to dysfunction of nitrated proteins, as has been shown or suggested in the case of superoxide dismutase, cytoskeletal actin, neuronal tyrosine hydroxylase, cytochrome P450 and vascular prostacyclin synthase. Oxidation of critical sulfhydryl groups is responsible for the inhibition of mitochondrial and cytosolic aconitase and other critical enzymes in the mitochondrial respiratory chain and disruption of the zinc-thiolate center at the active site of enzymes.

Peroxynitrite has been shown to inhibit a variety of ion pumps including calcium pumps, calcium-activated potassium channels and also membrane Na/K ATP-ase activity. These effects are likely to contribute to a global dysregulation of ion balance and a variety of related cellular functions.

The reaction of peroxynitrite with lipids leads to peroxidation (malondialdehyde and conjugated diene formation) and formation of nitrito-, nitro-, nitrosoperoxo- and/or nitrated lipid oxidation adducts.

Peroxynitrite can directly oxidize various biomolecules. Peroxynitrite-mediated oxidation of tetrahydrobiopterin (BH_4) to quinonoid 5,6-dihydrobiopterin has been demonstrated *in vitro*. A large proportion of the quinonoid isomer readily loses its side chain to form 7,8-dihydropterin which is not a cofactor for nitric oxide synthase. Thus, in endothelial cells and other cell types, pathophysiologically low levels of BH_4 can promote a cycle of its own destruction mediated by nitric oxide synthase-dependent formation of peroxynitrite.



Representative scheme of peroxynitrite-mediated cytotoxic processes and self-amplifying circles in various diseases. The reduction of oxygen supply during ischemia alters the mitochondrial function, leading to the production of reactive oxidant species. Superoxide and nitric oxide react to yield peroxynitrite. Peroxynitrite triggers a variety of oxidation, nitration and other cytotoxic processes, and enhances pro-inflammatory signalling via activation of the NF-κB pathway. Peroxynitrite and hydroxyl radical induce the single strand breakage in DNA, which, in turn, activates PARP. The activation of PARP rapidly depletes the cellular NAD^+ and ATP pools. The cellular energy exhaustion maintains the mitochondria in a reduced state, therefore allowing a further production of reactive oxidants at the reperfusion. Depletion of NAD^+ and ATP leads to cellular dysfunction. The cellular dysfunction is further enhanced by promotion of pro-inflammatory gene expression by via promotion of NF-κB, AP-1 and MAP kinase activation. Nitric oxide converts to the more cytotoxic nitroxyl anion (NO^-). Neutralization of peroxynitrite, oxyradicals and hydroxyl radical produces marked benefit in many experimental models of inflammation and reperfusion.

It is important to note that peroxynitrite-mediated inhibition of superoxide dismutase and other anti-oxidant molecules and systems. For example, peroxynitrite-mediated depletion of one of the key cellular anti-oxidants, glutathione can lead to positive feedback cycles of intracellular oxidant generation.

Oxidative stress may cause tissue injury through activation of the precursors of matrix metalloproteinase (proMMPs). The activation of proMMPs is triggered by peroxynitrite generation, via S-glutathiolation. By inhibiting this reaction, peroxynitrite decomposition catalysts reduce MMP activation, an important mechanism of tissue injury in inflammation and reperfusion.

An important interaction of peroxynitrite occurs with nucleic acids, with the production of 8-hydroxydeoxyguanosine or 8-nitroguanine.

The reactivity and decomposition pathways of peroxynitrite are strongly influenced by the chemical environment. In solutions containing carbonate, peroxynitrite forms an adduct with carbonate, which then may decompose to yield the toxic HCO_3 radical. In plasma, peroxynitrite oxidizes ascorbic acid, uric acid, tyrosine, and -SH groups of plasma proteins. Scavengers of peroxynitrite include uric acid, cysteine, glutathione, ascorbic acid, desferoxamine, Vitamin E and Trolox, its water-soluble analog and synthetic manganese-mesoporphyrins.

B.5.2. Peroxynitrite as a Therapeutic Target Peroxynitrite is more cytotoxic than NO or superoxide in a variety of experimental systems. In fact, recent studies suggest that, peroxynitrite, and not NO, may be the ultimately cytotoxic species in many conditions. For instance, *in vitro* studies demonstrate that NO itself has extremely limited effects on aconitase activity, whereas peroxynitrite is a much more potent inhibitor of aconitase under the same experimental conditions. Similarly, peroxynitrite, and not NO, is a potent initiator of DNA strand breakage.

In cells exposed to authentic peroxynitrite, marked changes in the level of cellular energetics and DNA integrity occur. For instance, in pulmonary type II cells, inhibition by peroxynitrite of membrane Na/K ATP-ase activity and sodium uptake has been reported. In neurons and glial cells, cultured monocyte-macrophages, cultured rat aortic smooth muscle cells, myocytes, endothelial cells and many other cell types a profound inhibition by peroxynitrite of mitochondrial respiration has been observed. While in neurons exposed to peroxynitrite, a decrease in the activity of NADH-COQ1 reductase, succinate-cytochrome c reductase and cytochrome c oxidase was found, in isolated mitochondria exposed to peroxynitrite, only cytochrome c oxidase was affected, suggesting the contribution of secondary cellular pathways to the toxicity of peroxynitrite.

Table 1. Selected cytotoxic processes initiated by peroxynitrite

On the molecular level

Action	**Mechanism**
Cytosolic enzyme inhibition	Oxidation, nitration
Membrane pump inhibition	Oxidation, nitration
Anti-oxidant enzyme inhibition	Oxidation, nitration
Signal transduction pathway disturbances	Oxidation, nitration
DNA injury	Oxidation, nitration, deamination, adduct formation
Metalloproteinase activation	S-glutoxidation of pro-metalloproteinases
Anti-oxidant enzyme depletion	Glutathione, cysteine oxidation
Inhibition of BH4 and NAD dependent enzymes	BH4 and NAD oxidation
Lipid peroxidation	Peroxidation, lipid peroxide chain reactions

On the subcellular level

Mitochondrial dysfunction	Inhibition of cytochromes, NADH-COQ1, etc.
NAD depletion	PARP activation, direct NAD oxidation
Upregulation of adhesion receptors	NF-kB activation
DNA fragmentation	DNA injury, caspase activation
Calcium dysregulation	Dysfunctional calcium pumps and cell energetics

On the cellular level

Necrosis	PARP activation, Mitochondrial injury, energetic collapse, oxidation, nitration, anti-oxidant depletion, calcium dysregulation
Apoptosis	Mitochondrial injury, DNA injury, caspase activation, signal transduction and calcium disturbances

It is very important in the context of radiation injury and subsequent auto-amplification mechanisms, that peroxynitrite – in addition to being a terminal mediator of cell injury – also enhances and triggers a variety of pro-inflammatory processes. For example, peroxynitrite enhances the expression of ICAM-1 and P-selectin in human endothelial cells, and it mediates the cytokine-induced IL-8 expression in human leukocytes. In human neutrophils, peroxynitrite triggers the down-regulation of L-selectin expression, and up-regulation of CD11b/CD18 expression. These effects are likely to be mediated, at least in part, by the ability of peroxynitrite to trigger and enhance nuclear factor kappa B (NF-κB) mediated pro-inflammatory signal transduction pathways. Peroxynitrite is also able to affect other signal transduction pathways, including protein kinase C) and MAP kinase. DNA single strand breakage, initiated by endogenous or exogenous peroxynitrite, is a potent trigger of poly(ADP ribose) polymerase (PARP) activation, which is a stimulator of base excision repair, but its overactivation can also be a major contributor to cell necrosis in many diseases.

Table 1 overviews some of the peroxynitrite-mediated deleterious molecular, subcellular and cellular pathophysiological alterations.

Peroxynitrite neutralizing agents are effective in many pathophysiological conditions associated with free radical and oxidant production (including stroke, myocardial infarction, arthritis, colitis and others). Some of them are listed in Table 2.

Table 2. The therapeutic potential of peroxynitrite neutralization

	Peroxynitrite formation demonstrated?	Demonstrated protective effect of NO or superoxide neutralization?	Protective effect of peroxynitrite neutralization?
Stroke	Yes	Marked protection	Not tested
Neurotrauma	Yes	Marked protection	Not tested
Myocardial infarction	Yes	Variable effects	Marked protection
Bypass surgery	Yes	Variable effects	Not tested
Hemorrhagic shock	Yes	Marked protection	Marked protection
Endotoxic shock	Yes	Marked protection	Marked protection
Bacterial sepsis	Yes	Marked protection	Marked protection
ARDS	Yes	Marked protection	Not tested
Toxic liver injury	Yes	Variable effects	Not tested
Reperfusion of gut	Yes	Marked protection	Protection
Diabetes	Yes	Marked protection	Marked protection
Diabetic complications	Yes	Marked protection	Marked protection
Arthritis	Yes	Marked protection	Marked protection
Colitis	Yes	Marked protection	Marked protection
Uveitis	Yes	Marked protection	Marked protection
Parkinson's disease	Yes	Marked protection	Not tested
Chronic heart failure	Yes	Variable effects	Protection
Doxorubicin cardiotoxicity	Yes	Variable effects	Marked protection

B.5.3. Peroxynitrite and radiation injury As (1) irradiation induces the generation of oxygen radicals; (2) most tissues in the body contain significant levels of nitric oxide under normal conditions; (3) the reaction of NO and superoxide occurs at a very fast reaction rate and (4) so far, practically in all diseases where oxyradicals are overproduced, the generation of nitrotyrosine (a footprint of peroxynitrite) has been demonstrated, one can hypothesize that peroxynitrite is also produced in response to whole-body irradiation. In addition, as shown in the subsequent section, WW-85, a peroxynitrite decomposition catalyst, is effective in protecting against mortality of mice subjected to whole-body irradiation. In addition, we have obtained data demonstrating the effectiveness of WW-85 against various forms of inflammatory and reperfusion diseases. These data are relevant to the current document, because the molecular mechanisms of free radical and oxidant mediated injury after irradiation and after ischemia-reperfusion are not dissimilar. In fact, anti-oxidant radioprotectants (e.g amifostine or melatonin) have also been demonstrated to be effective against various inflammatory and reperfusion diseases.

Recent work conducted in CHO cells subjected to irradiation demonstrated that one of the important positive feedback cycles (amplification mechanism) involves the stimulation of constitutive nitric-oxide synthase (NOS) activity. NOS activity of Chinese hamster ovary cells, as measured by the arginine --> citrulline conversion assay, was increased post-irradiation. Western blot analysis and genetic manipulation by overexpression of wild type or dominant negative NOS mutant identified the radiation-induced isoform as the constitutive NOS isoform. Protein tyrosine nitration, a footprint of peroxynitrite formation, followed radiation exposure was also shown, and this was inhibited by expression of a dominant negative NOS mutant. Radiation-induced ERK1/2 kinase activity, a cytoprotective response to radiation, was also blocked by inhibiting NOS activity, suggesting that peroxynitrite may activate this kinase pathway (as well as possibly other pathways of defense mechanisms and/or positive feedback cycles).

C. PRECLINICAL EFFICACY DATA

C.1. Medicinal chemistry background of Inotek's peroxynitrite catalyst program Inotek has been involved in the synthesis of potent catalytic anti-oxidants since 1999. One of the earlier generation prototypes, FP-15, proved to be highly potent peroxynitrite decomposition catalysts as well as SOD mimetics. The immediate challenges presented to us were characterization of both FP-15 and its synthetic intermediates and synthesis of the material in multi gram quantities. These issues have been resolved and FP15 has been generated and tested in multiple models of inflammation and reperfusion injury (e.g. Szabo et al., 2002; Mabley et al., 2002; Bianchi et al., 2002; Pacher et al., 2003).

While examining the PEGylation of the iron porphyrin intermediate (5,10,15,20- tetra(2-pyridyl)-21*H*,23*H*-porphine iron(III)), the use of other alkylating were tested in order to obtain general data on the alkyation of this intermediate. In doing so a series of species were generated with a variety of side chains on the pyridyl nitrogens. The biological activity of these compounds were tested and several examples exhibited better activity than the FP-15. Moreover, the analytical techniques developed for FP-15 were applied to these new compounds and showed that many side chains were less problematic than the PEG side arms of FP-15. Indeed, per-alkylation with a variety of alkylating agents yielded > 95 % of 4-arm species. From the library of N-substituted pyridyl porphyrins prepared, WW-85 demonstrated superior biological activities (see below) and was chosen as the candidate for further development.

The synthesis of WW-85 is via alkyation of 5,10,15,20- tetra(2-pyridyl)-21*H*,23*H*-porphine iron(III), so that the first two steps in the synthesis are the same as for FP-15. We have now developed clean-up procedures that remove the polymeric side products, which contributed about 80% of the bulk material isolated from this step. The column chromatography is still required, but on a scale about one tenth of that previously required. This is a major reduction in time, effort and generation of chemical waste. The insertion of the iron in the second step is relatively facile and is as described above (with minor modifications due to increased scale). The alkylation to WW-85 has been optimized. However, rotational isomers (atropoisomers, rotomers) are still produced and three major ones have been seen. Methods to separate these atropoisomers have been developed, and WW-85/Isomer #1, which exerts the highest biological activity and appropriate therapeutic window is now being made and tested routinely. The depth of knowledge gained in the process of understanding the chemistry of these types of compounds, and the analytic methods developed to meet the stringent requirements for a drug candidate, put Inotek in a unique position for the development of this type of therapeutic agent. From a synthetic and analytical chemical standpoint, we now have the know-how for reproducible scale-up and large-scale synthesis of the compound, as well as stability and forced degradation studies, and analytical and bioanalytical work.

C2. CHARACTERIZATION AND EFFICACY TESTING OF WW-85

WW-85 is water-soluble at concentrations of 20 mg/ml. Various formulations and routes of administration are under consideration, including intravenous, subcutaneous, aerosol and depot. WW-85 has limited oral bioavailability although preclinical models demonstrated efficacy even when administered orally.

WW-85 is a catalyst that reacts rapidly with peroxynitrite, hydrogen peroxide, and nitroxyl anion. Stopped-flow analysis reveals a reaction rate of $2x10^6$ mol sec^{-1} in the catalytic degradation of peroxynitrite. WW-85 does not scavenge nitric oxide and therefore does not interfere with NO mediated processes such as endothelium-dependent vasodilation. In primates, subcutaneous administration of WW-85 demonstrates a plasma half-life in excess of 20 hours.

In vitro assays have confirmed the cytoprotective activity of WW-85 (Fig. 1). These results were conducted using peroxynitrite, superoxide anion, nitroxyl anion and hydrogen peroxide models of cell death. Cell types studied include murine thymocytes, pancreatic beta islet cells, epithelial cells, and neuronal cells as well as various human cell lines. The *in vitro* therapeutic ratio was observed to be 1/100 as defined in cultured cells by minimally effective/minimally cytotoxic concentrations. Overall, we conclude that the most unique aspect of the compound is its ability to neutralize peroxynitrite, but the additional effects that the compound exerts on other oxidants and free radicals can produce additional therapeutic benefits (during radiation injury, as well as in other oxidant-mediated pathophysiological conditions). Below is a summary of the major preclinical *in vivo* and *in vitro* studies completed on WW-85 in 2004.

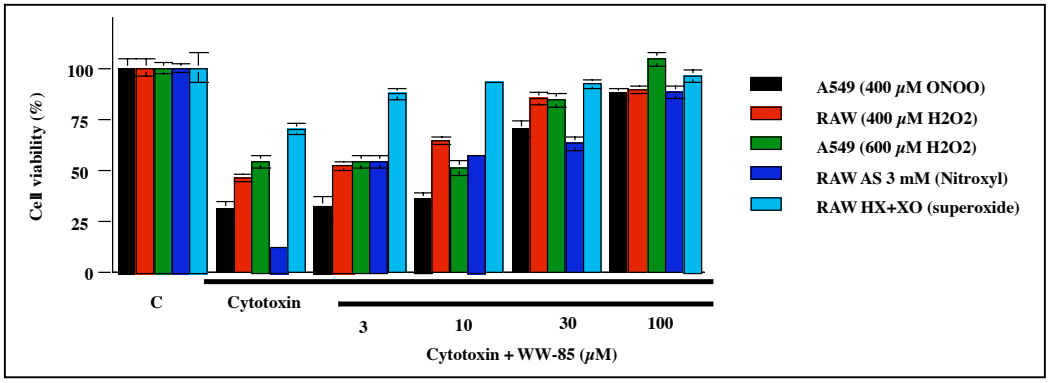


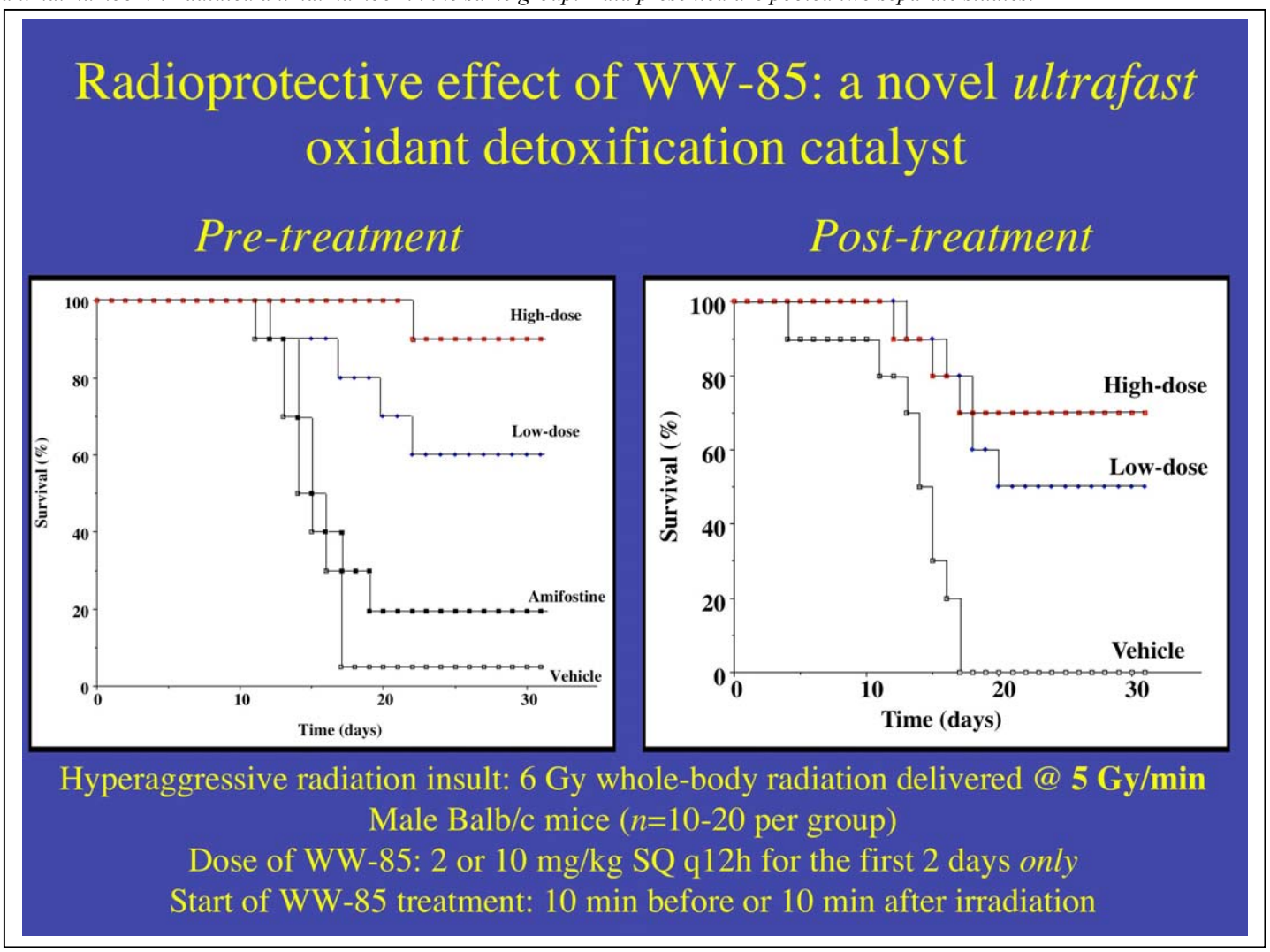
Fig. 1. *Suppression of mitochondrial respiration in human A549 and murine RAW cells by hydrogen peroxide, peroxynitrite, superoxide (generated from xanthine oxidase + hypoxanthine) and nitroxyl radical (generated by Angeli's salt): protection by 3- 100 μM WW-85.*

Multiple lines of evidence demonstrate that the compound WW-85 is effective in shock and reperfusion models *in vivo*. WW-85 was effective in a standard model of rat myocardial ischemia-reperfusion model, and it is most effective at a dose of 6 mg/kg. Subsequent studies are have been conducted to test the effect of the compound in rodent hemorrhagic shock models (Table 3). Hemorrhagic shock was used as a stringent and severe model system, to demonstrate efficacy of the compound, and to simultaneously characterize its mechanism of action on a variety of parameters (biochemical, cardiovascular, organ injury, survival). Additional studies demonstrated the efficacy of the compound in a wide variety of models that are governed by oxidant and free radical production (Table 3).

Table 3, Disease model	**Results**
Cytoprotection	
Cultured cell lines exposed to hydrogen peroxide, peroxide, superoxide, nitroxyl	Significant dose dependent cytoprotective effects
Cardioprotection (Ischemia Reperfusion)	
Banding induced heart failure model in mice	Inhibition of myocardial hypertrophy in response to chronic oral treatment
Myocardial Infarction in rats: LAD occlusion and reperfusion	Reduction in infarct size, reduction in circulating markers of myocardial necrosis and improved myocardial contractility
Radiation Protection	
Whole body irradiation studies in mice (6 Gy)	Improvement in survival rate in response to WW-85 pre-or post-treatment
Shock and Sepsis	
IL-2 induced shock in sheep	Improvement in all clinical outcome variables including hemodynamic stabilization and pulmonary function
Hemorrhagic shock in rats (fixed pressure model)	Improvement in blood pressure and survival; reduced organ injury; reduced tyrosine nitration and reduced TNF production
Diabetes	
Multiple low dose streptozotocin model of diabetes or nonobese diabetic spontaneous autoimmune diabetes	Significant reduction in diabetes onset and demonstration of preserved islet function.

D. PROGRESS REPORT: EFFICACY TESTING OF WW-85 IN MURINE MODELS OF RADIATION INJURY

Fig. 2. Protection against lethal irradiation by WW-85 treatment. *Male balb/c mice from Taconic were used. WW-85 was directly dissolved in 0.9% normal saline with appropriate concentration of WW-85 so that the final doses would be 2 or 10 mg/kg s.c. bid at 0.1ml/mouse. Treatment with WW-85 commenced 30 min prior to irradiation and continued for 2 days (4 subcutaneous injections, in total 8 or 40 mg/kg WW-85 over 2 days). The irradiation of 6 Gy (lethal dose in the present study series) was delivered by Gammacell 3000 Elan Irradiator from MDS Nordion(447 March Road, Kanata, Ontario, Canada). The survival ratio was calculated by survival animal number / irradiated animal number in the same group. Data presented are pooled two separate studies.*



We have generated significant data demonstrating the marked efficacy of WW-85 as a radioprotectant. The results demonstrated that WW-85 treatment provides a marked protection against radiation-induced mortality in an ultrasevere model of radiation injury induced by 6 Gy whole-body irradiation at an irradiation rate of 1 Gy over 12 sec. (Fig. 1). Reference compounds (300 mg/kg melatonin, 20 mg/kg amifostine) are virtually ineffective in the same experimental model, producing approximately 10-15% survival at 21 days.

The results shown in Fig. 2 were conducted using male Balb/c mice from Taconic. WW-85 was directly dissolved in 0.9% normal saline with appropriate concentration of WW-85 so that the final doses would be 2 or 10 mg/kg s.c. bid at 0.1ml/mouse. Treatment with WW-85 commenced 30 min prior to irradiation and continued for 2 days (4 subcutaneous injections, in total 8 or 40 mg/kg WW-85 over 2 days). WW-85 was administered in the morning (approx. 9 am) for the first injection and in the evening (approx. 5 pm) for the second injection.

In the pretreatment study, the first dose of WW-85 was given 30 minutes prior to the start of irradiation. In the post-treatment study, the first dose of WW-85 was given 10 min after irradiation.

The irradiation of the animals was delivered by a Gammacell 3000 Elan Irradiator from MDS Nordion (447 March Road, Kanata, Ontario, Canada). In the original calculations, the dose was calculated as 6 Gy. However, re-analysis of the radiation exposure demonstrates that the actual dose these studies used is 7 Gy. Radiation is being delivered at a rate of 6 Gy/min, which is an aggressive irradiation regimen.

The survival ratio was calculated by survival animal number / irradiated animal number in the same group. Data (*n*=10-20 animals for each experimental group) are presented in the figure. Data are pooled from two separate studies conducted in two separate experimental days (each study utilizing WW-85 treated animals, as well as its own internal vehicle controls).

Studies utilizing amifostine or melatonin In additional studies, animals treated with amifostine (10 mg/kg i.p.) or melatonin (300 mg/kg i.p.) were also studied. These compounds were given as a 30 min pretreatment in a single dose administration regimen. Amifostine or melatonin failed to affect survival, and survival rates in the treated groups were 10-20% (n=10 animals per group), whereas no animals survived in the vehicle-treated controls. Amifostine, at the very high dose of 200-400 mg/kg, however, did provide 90-100% survival benefit in our model. However, such high dosing with the compound is not achievable in humans, and therefore these findings have no practical relevance for development.

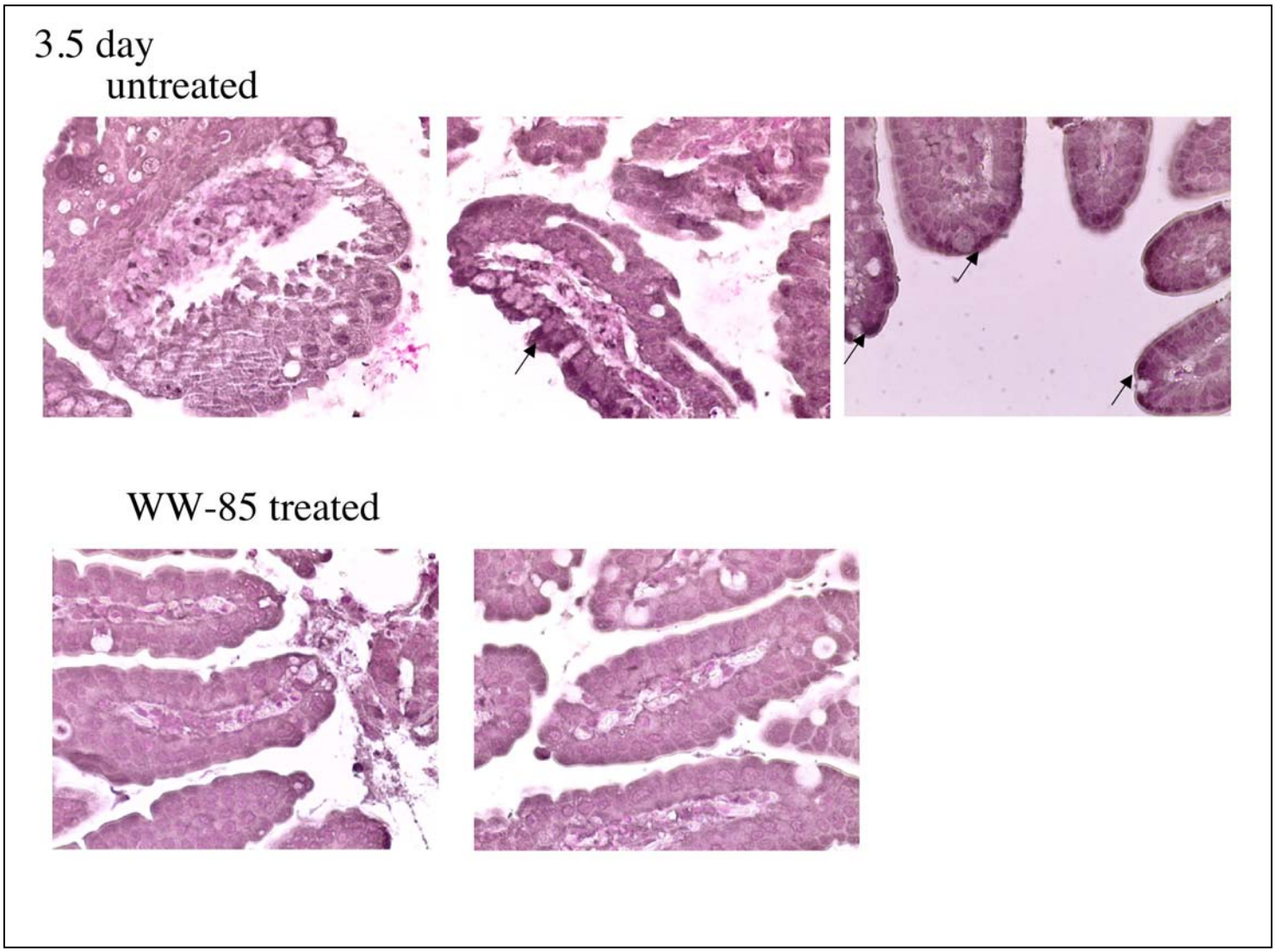
Additional efficacy and mechanistic studies with WW-85 Several studies are on-going with WW-85 to further characterize its radioprotective effect. In one study, we began to characterize the mode of radiation-induced mortality under our experimental conditions. We have observed no histological damage of the gut in irradiated animals (7 Gy) at 3.5 days after irradiation. On the other hand, we have observed a marked depletion of bone marrow cell counts at 5 days after the same dose of irradiation. Thus, we conclude that the current experimental model represent a marrow toxicity and immuno-suppression-based mortality model, as opposed to a gut failure-based model.

We also began to explore the effect of WW-85 on bone marrow toxicity. In the initial series of experiments (n=20 animals per group), control bone marrow values (number of granulocytes/ul) amounted to 16,360±1850. Irradiation (7 Gy) decreased these numbers to 622±66 at 5 days. Irradiated animals with WW-85 pretreatment (30 min prior radiation for the first dose of WW-85 at 10 mg/kg, followed by 3 additional doses for Days 1 and 2, as in the previous experimental designs) increased marrow cell counts to 1060±208 at 5 days ($p<0.01$ vs. irradiated). In irradiated animals with WW-85 post-treatment (first dose of WW-85 administered at 10 mg/kg at 30 min after irradiation, and subsequent 3 identical doses, as in the previous studies), cell counts were also partially maintained to 1431±408 ($p<0.01$ vs. irradiated). While WW-85 does not offer full protection against bone marrow granulocyte depletion, the doubling of cell count it offers may help protect against a critical degree of cell depletion, and it may be important in aiding the subsequent regeneration of circulating blood cells. On-going studies are going to address this question.

In several studies, we have explored the optimal dose of WW-85 (when given in 4 doses, over the first two days, the first dose being administered at a 30 min pretreatment). At 21 days, survival was 0% in vehicle-controls, 40% in 2 mg/kg x 4 group, 60% in 5 mg/kg x 4 group, 70% in 10 mg/kg x 4 group and 80% in 20 mg/kg x 4 group (n=10 animals /group).

In an additional series of experiments, we have explored the most optimal duration of WW-85 administration. We have compared the effect of a single dose WW-85 pretreatment (10 mg/kg) with two doses (one in a 30 min pretreatment at 9 am, one in post-treatment at 5 pm; at 10 mg/kg each), with four doses (as before, plus a third dose at 9 am and a fourth at 5 pm on the second day), with and six and eight doses (over 3 and 4 days, respectively). The results show that control animals had 10% survival rate at 21 days, animals treated with a single dose of WW-85 had 0% survival, animals treated with two doses had 30% survival, animals treated with four doses over 2 days had 90% survival, animals treated with six doses over 3 days had 80% survival and animals treated with eight doses over 4 days had 80% survival (n=10 per group). From these studies we conclude that the 10 mg/kg x 4 dosing (over 2 days) regimen provides the most optimal survival benefit in the current murine model. Because the half-life of WW-85 in mice is 20% of that in primates, we anticipate that a single SQ dose in man may be sufficient to provide continuous exposure to the drug for 48 hours (equivalent to the 2 day dosing regimen in mice).

Fig. 3. Protection against lethal irradiation induced tyrosine nitration by WW-85 treatment. *Male balb/c mice from Taconic were used. WW-85 was directly dissolved in 0.9% normal saline with appropriate concentration of WW-85 so that the final doses would be 10 mg/kg s.c. bid at 0.1ml/mouse. Treatment with WW-85 commenced 30 min prior to irradiation and continued for 2 days (4 subcutaneous injections, in total 8 or 40 mg/kg WW-85 over 2 days). The irradiation of 7 Gy (lethal dose in the present study series) was delivered by Gammacell 3000 Elan Irradiator from MDS Nordion(447 March Road, Kanata, Ontario, Canada).Please note the marked tyrosine nitration in the irradiated group (arrows depicting dark staining), and its reduction by WW-85 treatment. Data are representative sections from two separate sets of studies.*



Additional experiments, investigating the effect of WW-85 on tissue tyrosine nitration (an index of peroxynitrite generation) have also been conducted. The results (Fig. 3) demonstrate that radiation induces marked tyrosine nitration of the tissues, and WW-85 pretreatment attenuates this response.

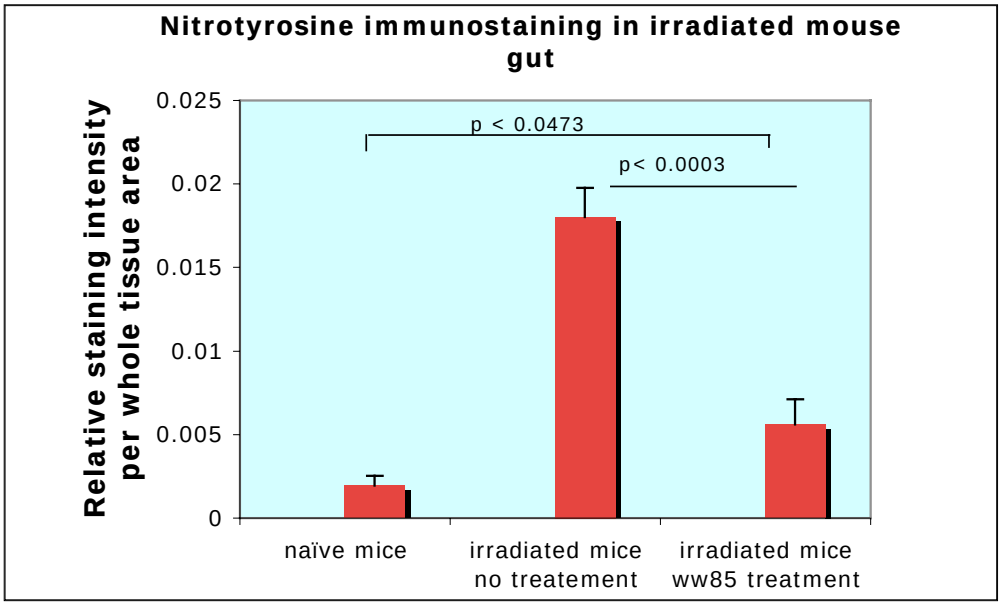


Fig. 4. Protection against lethal irradiation induced tyrosine nitration by WW-85 treatment. *Densitometric analysis of the studies described in Fig. 3.*

In an additional subset of studies, we have conducted *in vitro* studies in Hacat cells, in order to examine whether peroxynitrite sensitizes to the cytotoxic effect of irradiation (as no such studies have been conducted in the literature previously). We have also explored whether WW-85 treatment is able to protect against this potentiation. The results shown in Fig. 5 demonstrate that peroxynitrite potentiates radiation induced cell killing and WW-85 protects against this effect.

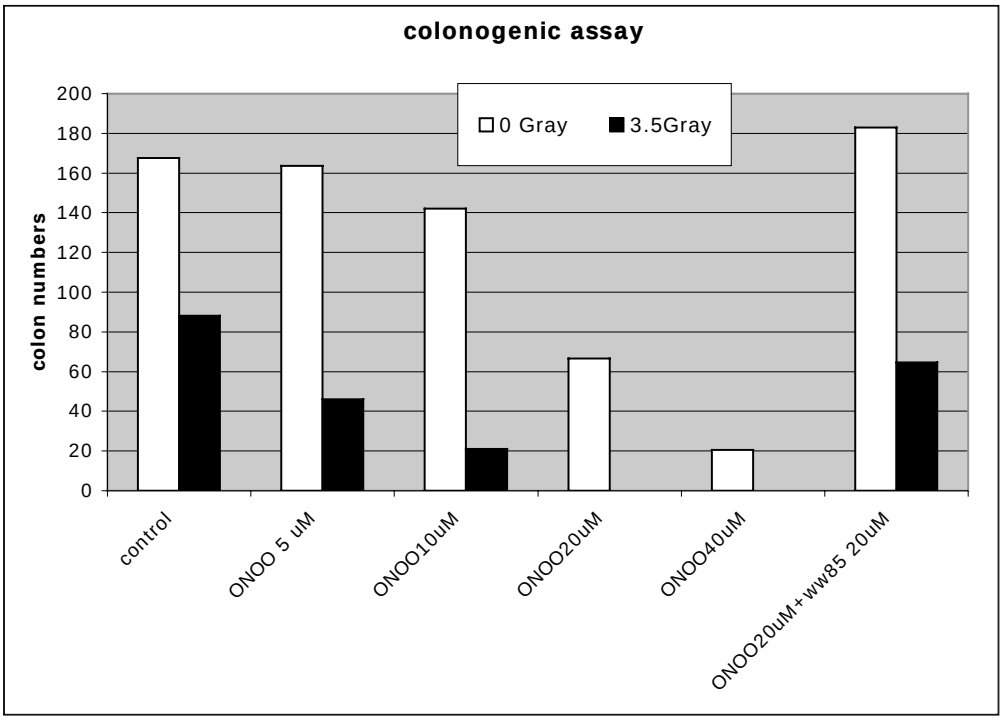


Fig. 5. Potentiation by peroxynitrite of radiation induced cell killing in Hacat cells, and protection by WW-85 treatment. *Clonogenic assays demonstrate that subthreshold concentrations of peroxynitrite markedly enhances the cytotoxic effect of 3.5 Gy radiation. The results also demonstrate that pretreatment of the cells with 20 uM peroxynitrite, coupled with 3.5 Gy radiation, induces complete cell killing, whereas WW-85 treatment in this situation offers ca. 50% cytoprotection.*

Overall, the studies demonstrated the radioprotective effect of WW-85 *in vitro* and *in vivo*. The doses of WW-85 have not produced any toxic effects in the animals, and plasma levels that are associated with protection against radiation induced mortality are readily achievable in primates (as shown by our pharmacokinetic studies).

In additional studies, we have conducted preliminary toxicology evaluation of the compound, where we have treated mice with doses of WW-85 identical to the radiation protective doses tested (2-10 mg/kg), as well as up to 10-30 times above. We have observed no mortality even at the dose of 300 mg/kg, and we have not observed significant increases in indices of organ damage (such as increases in hepatic enzymes or changes in renal function) (Fig. 6). These preliminary toxicity data are encouraging in terms of the commercial development of WW-85.

Studies in progress and studies planned for the future Additional studies are on-going (including evaluation of long-term survival rates from the above described studies, as well as determination of LD50 radiation values and effect of WW-85 treatment on the LD50). Additionally, a second, more aggressive irradiation protocol is being developed (12 Gy irradiation dose), where we expect to see a more rapid, gut-driven mortality, against which the effect of WW-85 is being tested. Finally, we have initiated the set-up of an attenuator, which will permit us to conduct additional studies at a lower rate of irradiation. Furthermore, additional studies, utilizing higher doses of WW-85 in a post-treatment therapeutic regimen are investigating the therapeutic window of opportunity with the compound (survival studies). Additional studies investigating the metabolism and pharmacokinetics of WW-85 in several species, as well as chemical scale-up and synthesis and toxicology studies have been initiated. In addition, we have began exploring possibilities to conduct primate or canine irradiation studies, in order to obtain proof of principle in a large animal model.

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